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# ChREBP binding to fatty acid synthase and L-type pyruvate kinase genes is stimulated by glucose in pancreatic $\beta$ -cells

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**Abstract** Pancreatic  $\beta$ -cell dysfunction is central to the pathogenesis of type 2 diabetes and may involve secretory failure through glucolipotoxicity. The relative importance of the transcription factors carbohydrate-responsive element binding protein (ChREBP), sterol-responsive element binding protein-1c (SREBP-1c), and upstream stimulatory factor (USF) in the induction of lipogenic genes by glucose remains unclear. By confocal imaging, we show that ChREBP translocates to the nucleus in MIN6  $\beta$  cells in response to glucose. Both ChREBP and SREBP-1c were required for the induction of the fatty acid synthase (FAS) promoter by glucose, and chromatin immunoprecipitation (ChIP) assay revealed that glucose induced the binding of both ChREBP and SREBP-1c to the FAS promoter without affecting USF2 binding. By contrast, ChIP assay revealed that high glucose prompted direct binding of ChREBP, but not SREBP-1c or USF2, to the liver-type pyruvate kinase (L-PK) promoter. This event was indispensable for the induction of the L-PK gene by glucose, as demonstrated by RNA silencing, single-cell promoter analysis, and quantitative real-time PCR. We conclude that ChREBP is a critical regulator of lipogenic genes in the  $\beta$  cell and may play a role in the development of glucolipotoxicity and  $\beta$  cell failure through alteration of gene expression in type 2 diabetes.—da Silva Xavier, G., G. A. Rutter, F. Diraison, C. Andreolas, and I. Leclerc. ChREBP binding to fatty acid synthase and L-type pyruvate kinase genes is stimulated by glucose in pancreatic  $\beta$ -cells. *J. Lipid Res.* 2006. 47: 2482–2491.

**Supplementary key words** glucolipotoxicity • lipogenic genes • chromatin immunoprecipitation

Carbohydrate response element binding protein (ChREBP, also known as WBSCR14 or Mondo B) (1) is a recently described transcription factor belonging to the basic helix-loop-helix leucine zipper family (2), which regulates carbohydrate metabolism in the liver (3). Mice deleted for both alleles of ChREBP (4) are glucose intolerant and in-

sulin resistant and have diminished rates of glycolysis and lipogenesis. This results in high liver glycogen content, low plasma free fatty acids levels, and reduced adipose tissue mass. Plasma insulin levels are normal despite elevated glucose concentrations, suggesting a defect in pancreatic  $\beta$  cell glucose sensing and/or insulin secretion.

In hepatocytes, ChREBP activity is regulated acutely by a series of phosphorylation/dephosphorylation reactions, although this has recently been challenged (5). At low glucose concentrations, ChREBP exists in an inactive, phosphorylated state in the cytosol. There are two major protein kinase A (PKA) phosphorylation sites, the first located near the nuclear localization signal at Ser<sup>196</sup> and the second near the DNA binding site at Thr<sup>666</sup>. Dephosphorylation of Ser<sup>196</sup> allows ChREBP entry into the nucleus, whereas dephosphorylation of Thr<sup>666</sup> allows DNA binding. AMP-activated protein kinase (AMPK) also regulates ChREBP by phosphorylation of Ser<sup>568</sup>, a modification that diminishes its DNA binding ability (6–8). Max-like protein X is the functional heteromeric partner of ChREBP in regulating the expression of glucose-responsive genes in hepatocytes (9).

Sustained hyperglycemia promotes fatty acid synthesis and triglyceride accumulation in the  $\beta$  cell. This “glucolipotoxicity” has been suggested by others (10), and ourselves (11, 12), to contribute to insulin secretory defects seen in type 2 diabetes. We have previously identified sterol-responsive element binding protein-1c (SREBP-1c) as an important transcription factor responsible for lipid accumulation in pancreatic islets in response to high glucose

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ChIP, chromatin immunoprecipitation; ChoRE, carbohydrate-responsive element; ChREBP, carbohydrate-responsive element binding protein; FAS, fatty acid synthase; L-PK, liver-type pyruvate kinase; PKA, protein kinase A; siRNA, small interfering RNA; SREBP-1c, sterol-responsive element binding protein-1c; TG, triglyceride; USF, upstream stimulatory factor.

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through stimulation of acetyl-CoA carboxylase-1 (ACC1) (11) and fatty acid synthase (FAS) (12) gene expression.

Because ChREBP is expressed in the pancreatic islets in a glucose-inducible manner, and forced overexpression of ChREBP in INS1 cells resulted in a left-shift of the liver-type pyruvate kinase (L-PK) gene in response to glucose (13), we have explored the role of ChREBP in the regulation of lipogenic genes in the  $\beta$  cell using both inactivation and overexpression strategies. Our results indicate that ChREBP binds directly to the endogenous promoters of the L-PK and FAS genes in a glucose-dependent but insulin-independent manner in MIN6 cells and is absolutely required for their induction by glucose. ChREBP is therefore potentially involved in the alteration of gene expression during the  $\beta$  cell compensation phase in the early stage of type 2 diabetes and could contribute to the development of  $\beta$  cell failure through lipopoptosis.

## MATERIALS AND METHODS

### Materials

The Silencer small interfering RNA (siRNA) construction kit was from Ambion (Huntingdon, UK). Primers for siRNA construction and PCR were from MWG Biotech (Milton Keynes, UK). TransIT<sup>TM</sup>-TKO transfection reagent was from Mirus Corp. (Madison, WI). Rat insulin radioimmunoassay kit was from Linco (St. Charles, MO). Tissue culture reagents were from Sigma-Aldrich (Dorset, UK) or Gibco-BRL (Paisley, UK). Lipofectamine 2000<sup>TM</sup> and SYBR Green platinum were from Invitrogen (Paisley, UK). Anti-upstream stimulatory factor-2 (anti-USF2) antibody was a kind gift from Dr. Benoît Viollet (Institut Cochin, Paris). Anti-SREBP-1c antibody was from Santa Cruz Biotechnology, Inc. Other reagents were from Sigma or BDH.

### Plasmids and adenoviruses

pChREBP was generated by RT-PCR from MIN6 cell total RNA using the following primers: 5'-ATG GAA CAG AAG CTT ATT TCT GAA GAA GAT CTT GCG CGC GCG CTG GCG GAT CTA TCC GTG AAC-3' (*c-myc* sequence underlined) and 5'-CGC GGA GCT CTT ATA ATG GTC TCC CCA GGG TGC CCT CTG TGA CTG C-3'. The resulting cDNA was phosphorylated by T4 polynucleotide kinase (Roche) and subcloned into pcDNA3 (Invitrogen) vector linearized with *EcoRV*. pFAS.Luc<sub>FF</sub> was generated by subcloning the *XhoI* fragment (−2187 to +65) of the rat FAS gene (14) into pGL3 basic (Promega) vector linearized with *XhoI*. All the constructs were verified by DNA sequencing. pLPK.Luc<sub>FF</sub> has been described previously (15), and plasmids encoding constitutively active and dominant-negative forms of SREBP-1c were as described in (11). Adenovirus encoding for the SREBP-1c dominant negative (SREBP-DN) control adenovirus expressing green fluorescent protein alone, and the infection procedure were as described (11).

### Generation of anti-ChREBP antibody

Polyclonal anti-ChREBP antibody was generated in rabbits immunized with a synthetic peptide corresponding to the C terminus of mouse and rat ChREBP [(C)ATRAVTEGTLGRPL], conjugated to keyhole limpet hemocyanin (KLH, Calbiochem) using sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce, Tattenhall, UK). Briefly, 10 mg KLH was gently dissolved in 2 ml PBS on a wheel rotator before

the addition of 2 mg Sulfo-SMCC and left on the wheel rotator for a further 3 h at room temperature. A Sephadex G-25 column (PD-10, Pharmacia Biotech) was equilibrated with 25 ml PBS, and the 2 ml KLH/Sulfo-SMCC mixture was loaded onto the column and eluted with 3.5 ml PBS. ChREBP peptide antigen (2.5 mg) was added to the eluate and left on a wheel rotator for 1 h at room temperature and overnight at 4°C. A Sephadex G-25 column was equilibrated with 25 ml H<sub>2</sub>O, loaded with the 3.5 ml peptide-antigen mixture and eluted with 3.5 ml H<sub>2</sub>O. The resulting conjugated peptide was stored at −20°C until further use. Immediately before rabbit immunization, 1 ml of conjugated peptide antigen was emulsified with an equal volume of Complete Freund's Adjuvant (Sigma). After taking preimmune serum from the rabbits, 800  $\mu$ l of the emulsion was injected per rabbit, subcutaneously, at four different sites (200  $\mu$ l/site) on the back, at week 0. Three booster injections in which the peptide antigen was emulsified with Incomplete Freund's Adjuvant (Sigma) were given as above at weeks 3, 6, and 9. Serum was taken at weeks 5 and 8, and a termination bleed was done at week 10. Purification of total IgG fraction was performed using caprylic acid and ammonium sulfate sequential precipitations, as described in (16).

### MIN6 cell culture

Cells were cultured in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. MIN6  $\beta$  cells (17) were used between passages 19 and 30 and grown in DMEM containing 15% (v/v) heat-inactivated fetal calf serum, 25 mM glucose, 5.4 mM KCl, 2 mM glutamine, 25  $\mu$ M  $\beta$ -mercaptoethanol, 100 IU/ml<sup>−1</sup> penicillin, and 100  $\mu$ g/ml<sup>−1</sup> streptomycin. MIN6 cells were seeded on poly-L-lysine-coated coverslips prior to microinjection. Plasmids and siRNAs were transfected into cells with Lipofectamine 2000<sup>TM</sup> and TransIT<sup>TM</sup>-TKO, respectively. Transduced cells were cultured in normal medium for 48 h and further cultured in medium containing 3 mM glucose 16 h prior to experiments.

### siRNA construction

siRNAs were generated using the Ambion Silencer siRNA construction kit according to the manufacturer's protocol. Starting primer pairs were designed as suggested in the Ambion protocol and according to published guidelines (18–20). Sense and anti-sense target sequences were derived from cDNA for ChREBP (GenBank accession number AB074517) with sense primer sequence as follows: 508-AAACCTGAGGCTGTTGTCTTCCTGTCTC-527 (T7 promoter sequence at the 3' end underlined), with the anti-sense primer having the complementary sequence. Control siRNAs were generated with primers derived by scrambling the siRNA sequence for ChREBP, with the sense primer sequence as follows: AAGGC-TGTTTCACCTGAGTTCCTGTCTC. All primer sequences were subjected to BLAST searches to ensure that there were no matches with known sequences of other genes. MIN6  $\beta$  cells were transfected with siRNA at a final concentration of 0.5 nM, according to the manufacturer's instructions and as described in (21).

### Western (immuno) blot analysis

Cells were washed twice in ice-cold PBS, scraped in ice-cold lysis buffer (PBS, 1% Triton X-100, 5  $\mu$ g/ml<sup>−1</sup> pepstatin, 5  $\mu$ g/ml<sup>−1</sup> antipain, 5  $\mu$ g/ml<sup>−1</sup> leupeptin, 2 mM benzamide and 0.5 mM dithiothreitol) and vortex-mixed. Protein content was assayed using a BCA<sup>TM</sup> protein assay kit (Pierce; Rockford, IL), against BSA Type V (Sigma) standards. Total protein extracts (50  $\mu$ g) were resolved by SDS-PAGE (10% w/v acrylamide) and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore), followed by immunoblotting with anti-ChREBP antibody used at 1:1000. Anti-rabbit secondary antibody (Amersham) was revealed

using BM chemiluminescence blotting substrate (Roche Diagnostics; Lewes, UK).

### Single-cell reporter gene assay

Intranuclear microinjection of plasmids, antibodies, and siRNAs was performed using an Eppendorf 5121/5246 micro-manipulator at plasmid concentrations of 0.1 (pLPK.Luc<sub>FF</sub>, pPdx1.Luc<sub>FF</sub>, and pFAS.Luc<sub>FF</sub>) and 0.05 (pCMV-RL) mg/ml<sup>-1</sup>, and antibody against ChREBP and SREBP at 1 mg/ml<sup>-1</sup> as described (15, 22). Antibody was injected into the nucleus and cytosol of the cell. Individual experiments involved injection of 100–200 separate cells per condition, with an efficiency of 5–20% productive injection as assessed by expression of *Renilla reniformis* luciferase activity. MIN6 cells were imaged 6 h after microinjection and culture under the conditions described in the figure legends. Photon counting imaging of firefly and *R. reniformis* luciferase activities was performed in single living cells using an Olympus IX-70 inverted microscope (×10 air objective, 0.4 NA) and an intensified charge-coupled device camera (Photek; East Sussex, UK) as previously described (15, 23).

### Insulin secretion

MIN6 cells, seeded in 6-well plates, were grown to 50% confluency and transfected with control or anti-ChREBP siRNA for 48 h (see above). Culture was continued for 16 h in DMEM containing 3 mM glucose. Cells were washed in PBS and incubated in Krebs Ringer Bicarbonate (KRB) medium containing either low (3 mM) or high (30 mM) glucose concentrations. Incubations were performed for 20 min at 37°C in a shaking water bath. Total and released insulin was measured by radioimmunoassay (24).

### Real-time RT-PCR

Total mRNA was isolated and specific messages quantified using TaqMan<sup>TM</sup> analysis as in (21), and TaqMan<sup>TM</sup> primers and internal probes or real-time primers (SYBR Green) for amplification of FAS,  $\beta$ -actin, and cyclophilin were as described in (21) and for L-PK as in (25).

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was performed as described in (26). MIN6  $\beta$  cells were cultured in medium containing 3 mM glucose for 16 h prior to stimulation with medium containing 3 or 30 mM glucose for 6 h prior to cross-linking with formaldehyde. DNA-protein complexes were immunoprecipitated with antibodies against ChREBP, SREBP-1c, and USF2 and then digested with proteinase K. After phenol-chloroform extraction and ethanol precipitation of the resulting DNA, PCR was performed to amplify the L-PK and FAS promoters. The primers used to amplify the mouse L-PK promoter from –392 to +94 bp were 5'-AAGTCTGCTTTAAGTGGGGCT-3' and 5'-TCCCCTCCAAGTTCCTCCAT-3'; for the FAS promoter from –135 to –14 bp, 5'-CGGCGCGCGGGTCCCGGG-3' and 5'-CCGCGCGCGGCTATTATAA-3'; and for the FAS promoter from –360 to –222 bp, 5'-CGCCCCGACCACTG-3' and 5'-CGGACGAAGGCAGGGGC-3'.

### Measurement of triglyceride content

Cells were transfected with control or ChREBP siRNA for 48 h, maintained for 16 h at 3 mM glucose, and then in 3 or 30 mM glucose for 24 h before measuring triglycerides (TGs) and protein concentrations. Total lipids were extracted using chloroform-methanol (2:1; v/v) as in (27). Extracted lipids were air dried, and 10 ml of a detergent (Thesit; Fluka, Gillingham,

Dorset, UK) was added to the dry pellet. Samples were air dried again and resuspended in 50  $\mu$ l H<sub>2</sub>O (28). TG was measured using a commercial kit (Infinity<sup>TM</sup> Triglyceride Reagent, Sigma) and a standard curve of triolein (Sigma) treated in parallel with the samples. Protein concentration was determined using a BCA kit (Pierce; Rockford, IL).

### Immunocytochemistry

Cells were fixed in 4% (v/v) paraformaldehyde and 0.3% Triton X-100 and stained with anti-*c-myc* antibody (9E10; 1:200), and revealed with fluorescein-conjugated goat anti-mouse IgG (Sigma) (22). Images were captured using a Leica SP2 laser scanning confocal microscope (×63 objective).

### Statistical analysis

Data are given as means  $\pm$  SEM. Comparisons between means were performed by unpaired one-tailed Student's *t*-test with Bonferroni correction as appropriate, using Microsoft Excel.

## RESULTS

### Elevated glucose concentrations cause nuclear translocation of ChREBP in MIN6 $\beta$ cells

To visualize the subcellular localization of ChREBP at low and high glucose concentrations, the relatively well differentiated  $\beta$  cell line MIN6 was transfected with *c-myc*-tagged pChREBP expression vector and incubated for 24 h at 3 mM glucose and then at 30 mM glucose for a further 16 and 24 h before fixation and immunocytochemical analysis, Hoeschst staining, and confocal imaging as described in Materials and Methods (Fig. 1A, B). The proportion of cells expressing ChREBP in the nucleus increased from 31% to 55% after 16 h at high glucose and to 69% after 24 h.

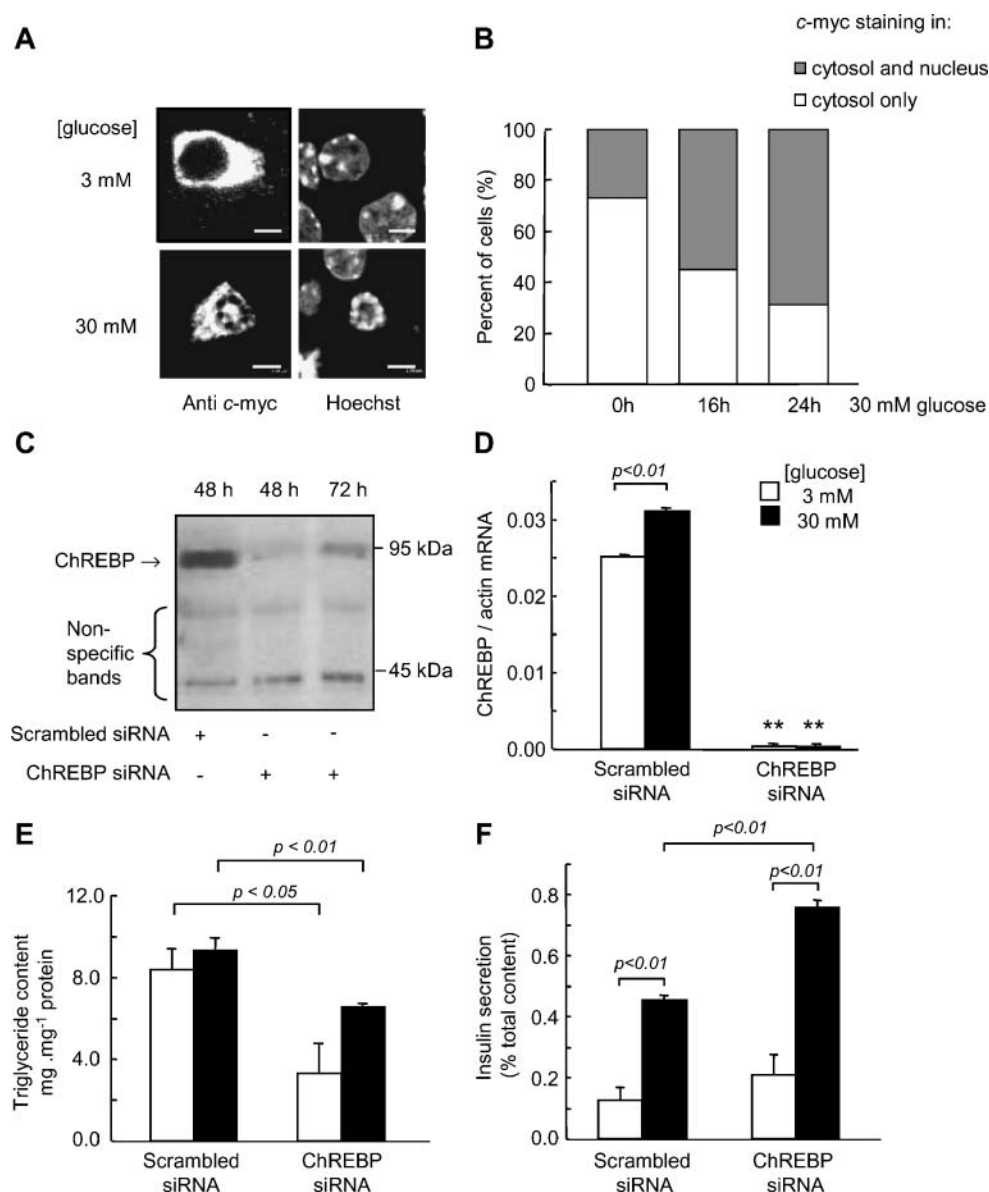
### ChREBP silencing decreases triglyceride content and potentiates glucose-stimulated insulin secretion in MIN6 cells

To determine the importance of ChREBP in the occurrence of glucolipotoxicity in MIN6 cells, ChREBP expression was silenced using siRNAs. ChREBP protein content was decreased by  $87.32 \pm 0.25\%$  and  $65.59 \pm 0.52\%$  at 48 and 72 h, respectively, as assessed by Western blot (Fig. 1C), and ChREBP message content was decreased by >95% at 48 h as assessed by quantitative real-time RT-PCR (Fig. 1D). ChREBP cellular depletion significantly decreased TG content at both 3 and 30 mM glucose concentrations (Fig. 1E) and slightly enhanced secreted insulin at 30 mM glucose (Fig. 1F).

### Both ChREBP and SREBP-1c are required for glucose-stimulated FAS gene expression in MIN6 cells

Given the decrease in MIN6 cellular TG content following ChREBP silencing (Fig. 1E), we next investigated the relative importance of ChREBP versus SREBP-1c in the regulation of lipogenic genes by glucose in this cell type. Figure 2 shows that both ChREBP and SREBP-1c are required for glucose-stimulated FAS gene expression in single MIN6 cells. Thus, the glucose responsiveness of the mouse FAS promoter was completely abolished following



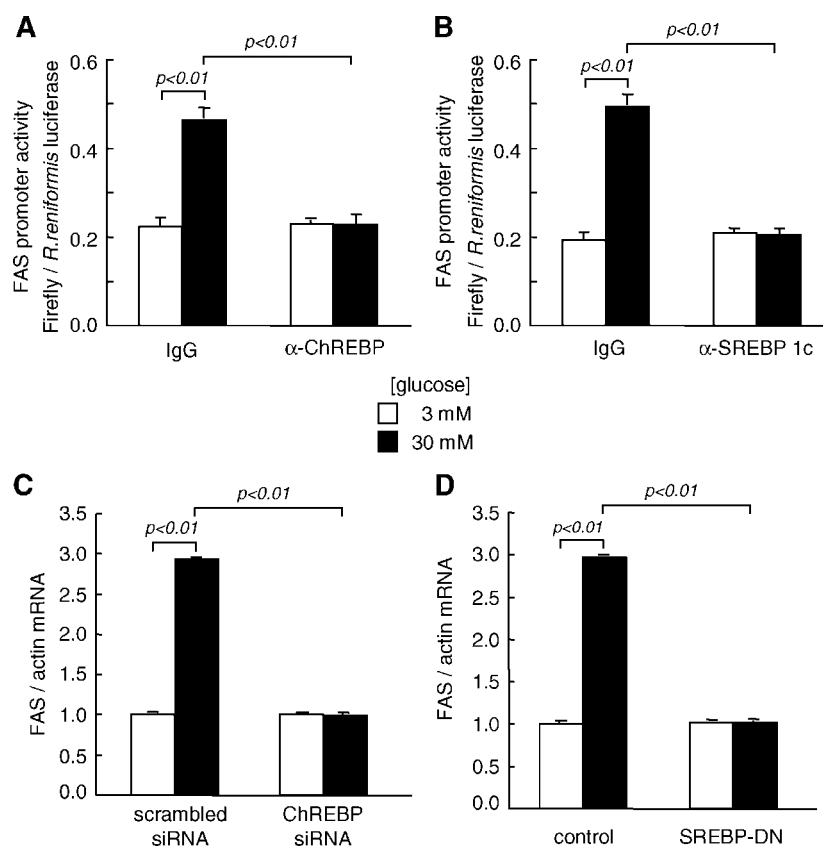


**Fig. 1.** Glucose regulates carbohydrate-responsive element binding protein (ChREBP) cellular localization and expression in MIN6 cells. **A:** MIN6 cells were seeded on poly-L-lysine-coated coverslips and transfected with pChREBP using Lipofectamine 2000<sup>TM</sup>. The cells were cultured in normal medium (25 mM glucose) for 24 h after transfection and then incubated in 3 mM glucose for 16 h and in either 3 or 30 mM glucose for a further 24 h, as indicated. ChREBP distribution and nuclear DNA were detected using anti-c-myc monoclonal antibody and Hoechst 33258 staining, respectively, prior to confocal imaging and cell counting (see Materials and Methods). **B:** Graphical representation of the experiment described in (A). **C, D:** MIN6 cells were transfected with ChREBP specific and scrambled small interfering RNAs (siRNAs) using TransIT<sup>TM</sup>-TKO and cultured in normal medium for 48 h or 72 h, as indicated, before Western blotting (C), or for 48 h in normal medium and 16 h in 3 mM glucose, followed with 6 h at 3 or 30 mM glucose, as indicated, before quantitative real-time RT-PCR assay (D). **E, F:** MIN6 cells were cultured as in (C) and triglycerides content and insulin secretion were performed as described in Materials and Methods. Data are means  $\pm$  SEM from a minimum of three separate experiments. \*\* $P < 0.05$  for the effect of specific versus scrambled siRNAs.

cellular microinjection of antibodies against either ChREBP (Fig. 2A) or SREBP-1c (Fig. 2B) together with pFAS.Luc<sub>FF</sub> and pRL.CMV constructs. Similarly, glucose induction of endogenous FAS mRNAs was inhibited in cell populations following ChREBP silencing using siRNA (Fig. 2C) or SREBP-1c inactivation with SREBP-1c-DN adenovirus (Fig. 2D).

### Glucose promotes the binding of ChREBP and SREBP-1c to the FAS promoter in vivo

Previous studies using SREBP-1<sup>-/-</sup> knock-out mice have shown the absolute requirement for this factor for the induction of the FAS gene by fasting/refeeding in the liver (29). Also, in USF1 and USF2<sup>-/-</sup> knock-out mice, the induction of FAS by fasting/refeeding was significantly

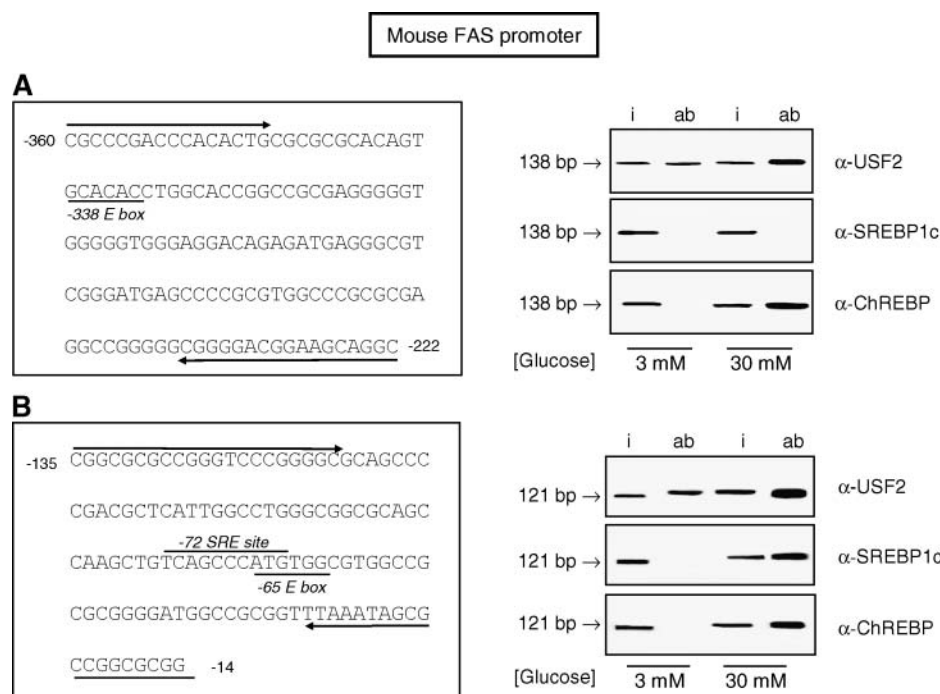


**Fig. 2.** ChREBP and sterol-responsive element binding protein-1c (SREBP-1c) are both required for glucose-induced fatty acid synthase (FAS) promoter activity. A, B: MIN6 cells were microinjected with antibodies against ChREBP ( $\alpha$ -ChREBP) (A), SREBP-1c ( $\alpha$ -SREBP-1c) (B), or normal IgGs, as indicated, together with FAS promoter-driven firefly luciferase reporter gene construct (pFAS.LucFF) and CMV promoter-driven *Renilla reniformis* luciferase reporter gene construct (pRL.CMV) as internal standard. The cells were cultured for 24 h in normal medium after microinjection, then switched to 3 mM glucose for 16 h and incubated for a further 6 h at 3 or 30 mM glucose, as indicated, before imaging and photon counting. C: MIN6 cells were transfected with anti-ChREBP specific or scrambled siRNAs, as indicated, and cultured as in Fig. 1D before quantitative real-time RT-PCR assay using SYBR Green as described in Materials and Methods. D: MIN6 cells were infected with SREBP dominant negative (SREBP-DN) adenovirus for 24 h before culturing in 3 mM glucose for 16 h followed by 6 h at 3 or 30 mM glucose, as indicated, before quantitative real-time RT-PCR assay using SYBR Green. Data are means  $\pm$  SEM from a minimum of three separate experiments.

impaired (30). To determine in the living pancreatic  $\beta$  cell the respective roles of ChREBP, SREBP-1c, and USF in the glucose responsiveness of the FAS gene, we performed chromatin immunoprecipitation assays using cells incubated at either low or high glucose concentrations and anti-USF2, anti-SREBP-1c, or anti-ChREBP antibodies. In the rat FAS promoter, because two E-boxes, located at  $-332$  and  $-65$  bp, bind USF *in vitro* (31), we used two sets of PCR primers that would include these sites in the mouse FAS promoter (arrows in Fig. 3A, B, left panels). As shown in Fig. 3, USF2 bound at both 3 and 30 mM glucose, to both the proximal and distal stretches of sequences included in our chromatin immunoprecipitation (ChIP) assay. On the other hand, SREBP-1c was bound to the proximal stretch of sequence at high glucose concentrations only. ChREBP, however, bound to both stretches of the promoter in a strongly glucose-dependent manner (Fig. 3A, B).

### ChREBP, but not SREBP-1c, is responsible for the glucose responsiveness of the L-PK gene in MIN6 cells

Inhibition of endogenous ChREBP through the introduction of an antibody against ChREBP into MIN6 cells by microinjection led to ablation of glucose-induced L-PK promoter activity (Fig. 4A), whereas overexpression of the wild-type protein did not cause a significant change at basal glucose concentration but led to a  $1.75 \pm 0.007$ -fold increase at 30 mM glucose (Fig. 4B). ChREBP knockdown by RNA silencing led to a concomitant ablation of glucose-induced changes in L-PK mRNA levels (Fig. 4C), as assessed by real-time quantitative RT-PCR. Because we have previously reported (32) that glucose induction of the L-PK promoter in the pancreatic  $\beta$  cell was secondary to enhanced insulin secretion, and that insulin was sufficient to activate L-PK transcription, we next investigated whether ChREBP silencing would interfere with insulin activation of L-PK transcription. Indeed, as shown in Fig. 4D, the in-



**Fig. 3.** ChREBP and SREBP-1c directly bind to the FAS promoter in a glucose-dependent manner in vivo. A, B: MIN6 cells were incubated in 3 mM glucose for 16 h and for a further 6 h at 3 or 30 mM glucose, as indicated, before a chromatin immunoprecipitation (ChIP) assay was performed as described in Materials and Methods using antibodies against upstream stimulatory factor-2 (USF2) ( $\alpha$ -USF2), SREBP1-c ( $\alpha$ -SREBP1-c), or ChREBP ( $\alpha$ -ChREBP), as indicated in the figures. The left panels show the primers used (arrows) and the sequence amplified by PCR. The consensus E-boxes and sterol-responsive element site are also indicated. The right panels show the resulting PCR products using the input fractions (i) and following immunoprecipitation with the specific antibodies (ab) at either 3 or 30 mM glucose. Data are representative of three independent experiments.

duction by insulin of L-PK mRNA at low glucose concentration was blunted following ChREBP knockdown. On the other hand, overexpression of the constitutively active nuclear fragment of SREBP-1c had no effect on L-PK promoter activity (Fig. 4E), nor did the overexpression of dominant negative SREBP-1c affect L-PK mRNA levels (Fig. 4F).

#### ChREBP, but not SREBP-1c, binds to the L-PK promoter in a glucose-dependent but insulin-independent manner in living cells

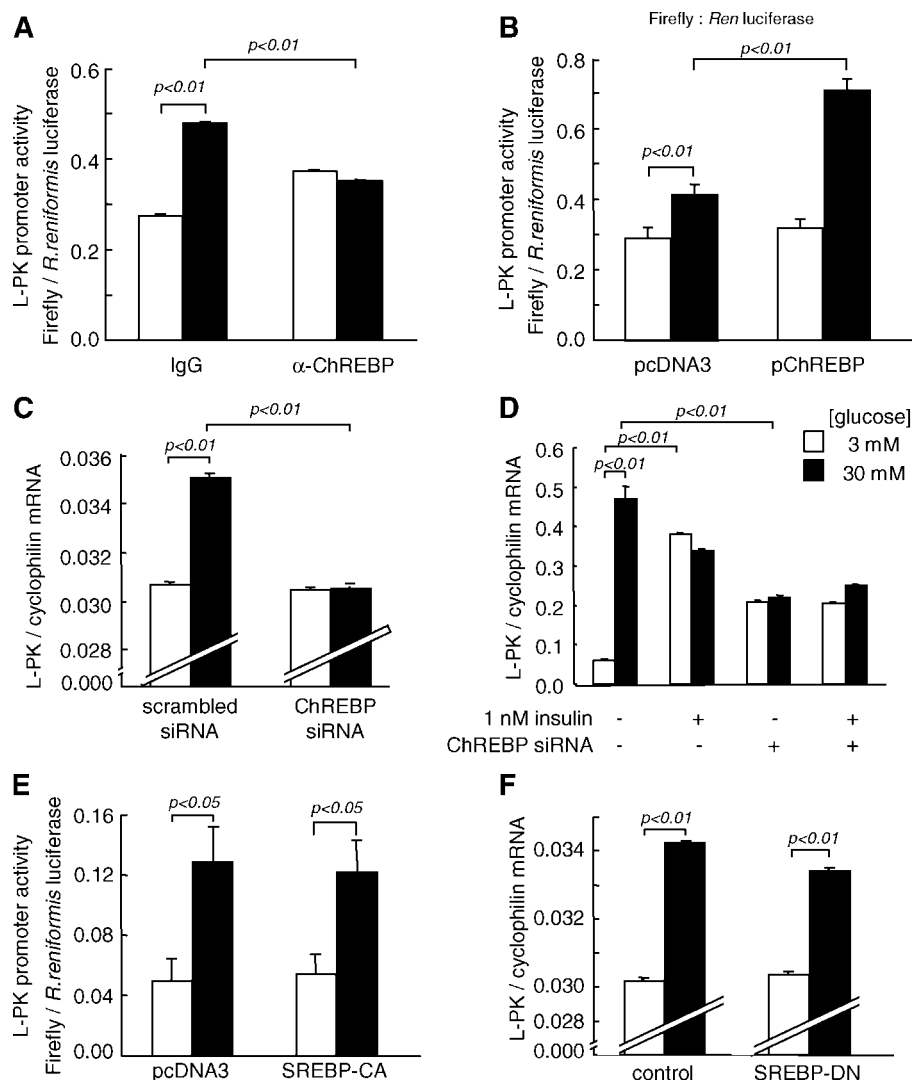
The carbohydrate-responsive element (ChoRE) of the L-PK promoter is well characterized and is centered at -177 bp on the mouse promoter (Fig. 5A, underlined) (33). ChIP assay revealed that ChREBP bound directly to this segment of the L-PK promoter at 30 but not 3 mM glucose. In contrast, chromatin immunoprecipitation using anti-USF2 or anti-SREBP-1c antibodies failed to pull down this segment of the L-PK promoter (Fig. 5B). To determine whether insulin stimulated L-PK promoter activity directly through ChREBP binding, we performed a ChIP assay using ChREBP antibody at low and high glucose, and in the presence or absence of 1 nM insulin, or while inhibiting insulin release with the hyperpolarizing agent diazoxide. As shown in Fig. 5C, the addition of 1 nM insulin to MIN6 cells cultured at low glucose did not cause any increase in ChREBP binding, although this maneuver

resulted in the stimulation of L-PK gene expression as assessed by quantitative RT-PCR (Fig. 4D). However, inhibition of glucose-induced insulin secretion with 100  $\mu$ M diazoxide completely abolished ChREBP binding at high glucose (Fig. 5C).

#### DISCUSSION

##### ChREBP is a key regulator of lipogenic genes in the $\beta$ cell

We have previously demonstrated that SREBP-1c is an important regulator of lipogenesis in  $\beta$  cells (11, 12). Thus, overexpression or inactivation of SREBP-1c strongly modulates the ACC1 (pII) promoter (11). We show here that SREBP-1c inhibition, achieved through antibody microinjection of adenoviral overexpression of SREBP-DN, also abolishes the induction by glucose of FAS promoter and mRNA levels in MIN6 cells (Fig. 2). By contrast, SREBP-1c overexpression and inhibition had no effect on L-PK transcription at either low or high glucose concentrations (Fig. 4E, F), implying a role for other transcription factor(s) in the response of the latter gene to glucose. ChREBP is emerging as a key regulator of lipogenic genes in the liver. Very recently, ChREBP-deficient mice were intercrossed with *ob/ob* mice, and the resulting offspring had a better metabolic profile, as well as decreased ap-



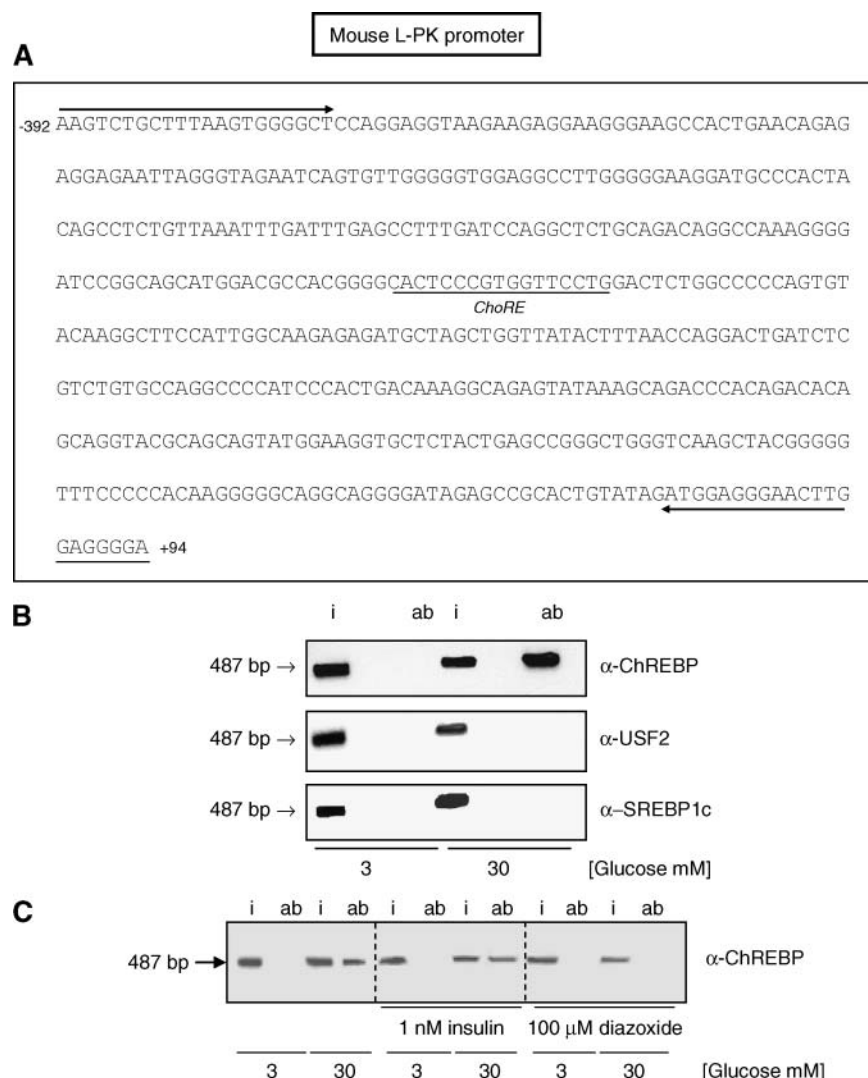
**Fig. 4.** ChREBP, but not SREBP-1c, mediates glucose responsiveness of the liver-type pyruvate kinase (L-PK) promoter in MIN6 cells. A, B, E: MIN6 cells were microinjected with normal IgG or anti-ChREBP antibodies ( $\alpha$ -ChREBP) (A), with pcDNA3 or pChREBP (B), or SREBP-CA (E), as indicated, together with pLPK.LucFF and pRL.CMV plasmids before incubation and photon counting as in Fig. 2. C, D, F: MIN6 cells were transfected with scrambled or specific ChREBP siRNAs (C, D) or infected with control or SREBP-DN adenovirus (F), cultured for 24 h in normal medium, then incubated in 3 mM glucose for 16 h and stimulated with 3 or 30 mM glucose, as indicated, for another 6 h before RNA extraction and real-time RT-PCR assay using TaqMan, as described in Materials and Methods. Data are means  $\pm$  SEM from a minimum of three separate experiments.

petite and adiposity, suggesting a role for ChREBP in peripheral tissues, including the brain (34). We show here that overexpression of ChREBP potentiates the effects of glucose on the L-PK promoter in  $\beta$  cells (Fig. 4B), consistent with previous findings in INS-1 cells (13) as well as hepatocytes (3). However, we now report for the first time that inactivation of ChREBP, achieved by RNA silencing or antibody microinjection, inhibits the induction of L-PK by glucose in  $\beta$  cells (Fig. 4A, C).

Using direct ChIP assay, we also provide the novel observation that in MIN6 cells, glucose signaling promotes binding of ChREBP to the carbohydrate response element of the L-PK promoter. By contrast, neither SREBP-1c nor USF binding to this site was observed at either high or low

glucose concentrations (Fig. 5). These findings would appear to question earlier results from this laboratory implicating USF as an important regulator of the L-PK gene in  $\beta$  cells (15), possibly reflecting the uncertain specificity of the antibodies used in these earlier microinjection studies, but they are consistent with the findings of Kaytor, Shih, and Towle (35), who argue against a role for USF in the regulation of L-PK gene expression in the liver, and the findings of Wang and Wollheim (13) in INS-1 cells. We propose that in  $\beta$  cells, the absence of ChREBP binding to the L-PK ChoRE at low glucose concentrations may be explained by both nuclear exclusion (Fig. 1A) and posttranscriptional modifications, as in hepatocytes (6, 8). However, in contrast to the liver system, the actions of






**Fig. 5.** ChREBP binds to the L-PK promoter in a glucose- and calcium-dependent but insulin-independent manner in vivo. MIN6 cells were incubated for 16 h at 3 mM glucose and then for 6 h at either 3 or 30 mM glucose, in the presence or absence of 1 nM insulin and 100 μM diazoxide, as indicated, before cross-linking and ChIP assay as in Fig. 3. A: The primers (arrows) and the amplified sequence of the mouse L-PK promoter by PCR are shown. The carbohydrate-responsive element is underlined. B, C: Shown are the resulting PCR products following immunoprecipitation using anti-USF2 (α-USF2), anti-SREBP (α-SREBP), or anti-ChREBP (α-ChREBP) antibodies as indicated. i, input fraction; ab, antibody fraction. Data are representative of three independent experiments.

glucose on L-PK expression in MIN6 cells are in large part mediated by the secretion of insulin (32). Although insulin is not sufficient to promote ChREBP binding to the L-PK promoter in vivo (Fig. 5C), insulin stimulates the L-PK promoter at low glucose concentrations and ChREBP is necessary for insulin stimulation of the L-PK promoter (Fig. 4D). Also, inhibiting glucose-induced insulin secretion by the use of diazoxide prevents ChREBP binding on the L-PK promoter (Fig. 5C). The latter observation could suggest a role for changes in intracellular free  $\text{Ca}^{2+}$  concentrations in the activation of ChREBP, although more experiments are needed to confirm this hypothesis. Alternatively, insulin could be necessary for binding or activation of a distinct transcription factor that works in conjunction with ChREBP. Further work is required to

determine the respective roles of intracellular glucose metabolism, insulin release, and the activation or inhibition of protein kinases [e.g., PKA, AMPK, Calmodulin Kinase kinase (CamKK)] in modulating the subcellular localization of ChREBP and its transcriptional activity in the pancreatic  $\beta$  cell.

In contrast to the L-PK gene, we demonstrate here that both ChREBP and SREBP-1c are necessary for the glucose responsiveness of the FAS promoter (Fig. 2). Moreover, both ChREBP and USF2 bound to both stretches of the FAS promoter examined (containing the -65 and -332 E-boxes, Fig. 5), ChREBP in a glucose-dependent manner and USF2 constitutively. The apparent increase in intensity following immunoprecipitation with anti-USF2 observed at high glucose was not always observed between

our replicates. SREBP-1c also bound to the FAS promoter in response to elevated glucose concentrations, but only to the more proximal segment containing the -65 E-box. The elegant work of Sul et al. (36), using in vivo chromatin immunoprecipitation from extracts of livers of transgenic mice harboring various truncations and mutations of an FAS promoter CAT reporter gene construct, showed that SREBP-1c binds only to the -150 sterol response element (SRE) and not to the -65 region containing an E-box and an overlapping SRE (shown in Fig. 3B). The resolution of the ChIP method is largely dependent on the fragment size obtained on shearing the chromatin, rather than on the primer sites chosen for PCR. Although the -150 SRE site is theoretically excluded from our ChIP assay, it is located just outside our -135 primer and may have been pulled down during immunoprecipitation using the SREBP1 antibody. It is also possible that the transcription factor recruitment might be different in  $\beta$  cells compared with liver.

Taken together, these results suggest an important role for ChREBP in  $\beta$  cell failure due to lipid accumulation secondary to chronic high glucose exposure through activation of lipogenic genes, because ChREBP silencing decreases triglyceride content at both basal and elevated glucose concentrations (Fig. 1E), and increases insulin secretion at high glucose concentrations (Fig. 1F). However, although MIN6 cells are a nice in vitro model, more work is needed to confirm a role for ChREBP in glucolipotoxicity in primary islets in vivo. 

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